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Rapid Communication

The stability of the human papillomavirus E6 oncoprotein is E6AP dependent

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ABSTRACT

Human papillomavirus (HPV) E6 oncoproteins target numerous cellular proteins for ubiquitin-mediated degradation. In the case of p53 this is mediated by the E6AP ubiquitin ligase. However, there are conflicting reports concerning how central E6AP is to the global function of the HPV-16 and HPV-18 E6 oncoproteins. To investigate this further we have analysed the effects of E6AP removal upon the stability of endogenously expressed E6 protein. We show that when E6AP is silenced in HPV-positive cells, E6 protein levels are dramatically decreased in a proteasome-dependent manner. Further, we show that when E6AP is depleted in HeLa cells, E6 has a greatly decreased half-life. In addition, overexpression of E6AP stabilises ectopically expressed HPV-16 and HPV-18 E6 in a manner that is independent of its ubiquitin ligase activity. These results demonstrate that the stability of HPV E6 is critically dependent upon the presence of E6AP.

Introduction

The oncogenic activity of high-risk HPV is caused by cooperation of two viral oncoproteins, E6 and E7. They target cellular proteins involved in the regulation of apoptosis and cell cycle, causing immortalization and, eventually, cellular transformation (Mantovani and Banks, 2001; Munger et al., 2001). One of the most important targets of E6 is the p53 tumour suppressor, which it targets for proteasome mediated degradation (for review Thomas et al., 1999). This activity is dependent upon the recruitment of a 100 kDa cellular ubiquitin ligase, E6AP, to the complex, which then leads to p53 poly-ubiquitination followed by proteasomal degradation (Huibregtse et al., 1993; Scheffner et al., 1993).

Many studies have shown that in the absence of E6AP, E6 is no longer able to target p53 for degradation, and this leads to an increase in p53 levels and activity (Beer-Romero et al., 1997; Hengstermann et al., 2005; Talis et al., 1998). However, whether E6AP plays a central role in other activities of E6 has been the subject of considerable debate. Many *in vivo* analyses highlight the importance of E6AP for E6 mediated degradation of several cellular targets (Handa et al., 2007; Kuballa et al., 2007), including the PDZ domain-containing substrates, whilst other *in vitro* analyses seem to suggest that E6AP is not always required (Grm and Banks, 2004). A number of recent studies have also suggested that E6 can even target p53, as well as other substrates, in an E6AP-independent fashion *in vivo* (Camus et al., 2007; Grm and Banks, 2004; Massimi et al., 2008; Shai et al., 2007; Storrs and Silverstein, 2007). Finally, an elegant series of studies has suggested that the global transcriptional effects of E6 upon the cell are largely dependent on the presence of E6AP (Kelley et al., 2005), suggesting, indirectly, that all of E6's activities are mediated through its association with E6AP.

Results

E6 protein stability is E6AP dependent in HeLa cells

Many of the above studies have involved either overexpression systems or, in cases where endogenous E6 was analysed, under conditions in which the level of E6 protein expression was not determined. Therefore in order to further clarify the role of E6AP in the function of E6, we first investigated the levels of E6 expression in HPV-positive cells in the presence and absence of E6AP. To do this, HPV-18-containing HeLa cells were transfected with siRNAs directed against either E6AP, HPV-18 E6 or Luciferase for comparison, and the levels of both proteins were then analysed by western blot analysis. The proteasome inhibitor CBZ was also added to the cells 2.5 h prior to harvesting to determine whether changes in the pattern of protein expression were proteasome-dependent. Cells were harvested 72 h post-transfection, and the total cellular lysates were subjected to western blot analysis using anti-E6AP, anti-tubulin, and anti-18E6 antibodies. The results obtained are shown in Fig. 1. As can be seen, the reduction of E6 levels by siRNA results in a marked upregulation in the level of E6AP expression, and this is consistent with previous observations showing that E6 induced the auto-ubiquitination and degradation of E6AP (Kao et al., 2000). Most strikingly however, ablation of E6AP expression also results in a dramatic reduction in E6 protein, to levels that are even lower than those obtained using the E6 siRNA. In addition, inclusion of the proteasome inhibitor CBZ prior to harvest rescued the expression of E6, indicating that its decreased level in the cell is proteasome mediated. In order to reduce the possibility of off-target effects of

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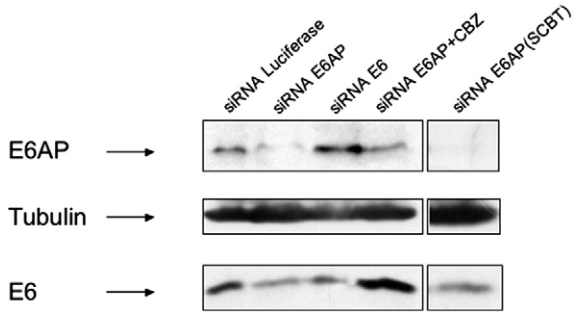

Fig. 1. HPV E6 protein stability in HPV-positive HeLa cells is E6AP dependent. HeLa cells were transfected with siRNA Luciferase, or siRNA E6AP, or siRNA 18E6/E7. After 72 h cells were incubated with or without CBZ (Z-Leu-Leu-al/Sigma) for 2.5 h to block the proteasome, with DMSO treatment as control. The cells were then harvested and the protein levels were detected using western blotting with anti-E6AP antibody, anti-tubulin antibody to monitor protein loading, and anti-18E6 antibody, followed by HRP-coupled anti-mouse antibody and ECL detection. Note that the siRNA E6AP (SCBT) track is from the same experiment and western blot and the arrows indicate the positions of the E6AP, tubulin, and E6 proteins.

the E6AP siRNA, we repeated the analysis using a different siRNA from another supplier (SCBT lane), and an identical reduction in the level of E6 expression was obtained. These results demonstrate that E6 steady state levels are strongly dependent upon the presence of E6AP, and that when E6AP is not present E6 is down-regulated in a proteasome-dependent manner.

E6AP silencing does not significantly affect E6 RNA transcription

To further investigate the effects of E6AP ablation upon E6 expression levels, we proceeded to determine whether this was in any way related to the level of E6 gene expression. To do this, HeLa cells were transfected with siRNA Luciferase or siRNA E6AP. After 72 h the cells were harvested and total RNA was extracted and processed with and without Reverse Transcriptase. The cDNAs were then amplified using HPV-18 E6 flanking primers and the results obtained are shown in Fig. 2. It can be seen that the presence or absence of E6AP has minimal effect upon the level of HPV-18 E6 gene expression, as determined by this semi-quantitative assay. These results demonstrate that the decrease in the levels of E6 protein that is observed when E6AP is depleted is not due to any major changes in the levels of HPV gene expression, but rather is mostly due to a decrease at the protein level.

E6AP regulates E6 protein turnover

Having shown that E6AP ablation reduces the steady state levels of E6 we then wanted to analyse the effects upon E6 protein turnover.

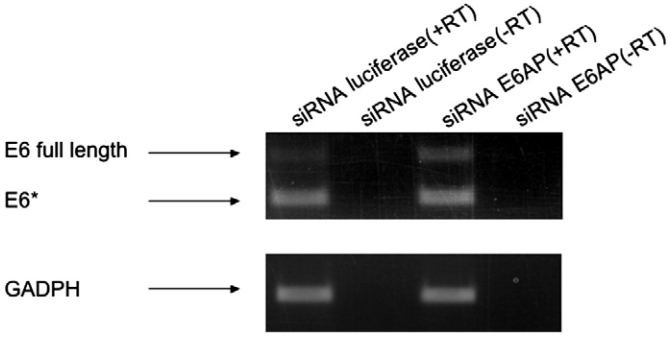

Fig. 2. E6 RNA transcripts are not affected by E6AP silencing. HeLa cells were transfected with siRNA Luciferase or siRNA E6AP. After 72 h cells were harvested and total RNA was extracted. Purified RNA was then annealed with random decamers and cDNAs generated with Reverse Transcriptase; to control for plasmid DNA carry over, a parallel set of random decamer-annealed samples was incubated without RT (− RT). The cDNAs generated from the RT step, and their control samples were amplified using HPV-18 E6 flanking oligos. Arrows indicate the position of full length E6, alternatively spliced E6* (Schneider-Gadicke and Schwarz, 1986) and the GADPH loading control.

Assays were performed as above in HeLa cells, using siRNA Luciferase as a negative control and siRNA against E6AP. 48 h post-transfection the cells were treated with Cycloheximide for different times in order to determine whether E6AP had any effect on E6 protein half-life. The levels of E6 protein were then assessed by western blot analysis using the anti-18 E6 monoclonal antibody and the results obtained are shown in Fig. 3A, with the quantitation from multiple assays shown in Figs. 3B and C. As can be seen, when cells were treated with the control siRNA the E6 protein levels remained relatively unchanged from time-point 0 to 60 minutes, and they started to decrease at the 120 minute time-point, as one would expect from previous reports (Androphy et al., 1987; Grossman et al., 1989). In contrast, when E6AP is depleted from the cells the E6 levels show a significant decrease by the 30 minute time-point, and the protein is completely absent by the 120 minute time-point. These results show that silencing E6AP in HPV-positive HeLa cells results in a dramatic increase in E6 protein turnover.

We then reasoned that under conditions of E6 overexpression, co-transfection of exogenous E6AP should also result in an increase in the levels of E6 protein expression. To investigate this, HEK 293 cells were transfected with HPV-16 and HPV-18 E6 expression plasmids, together with FLAG-tagged wild type E6AP and a catalytically inactive E6AP mutant, in order to determine whether E6AP enzymatic activity was also required for E6 stabilisation. The expression levels of E6AP and E6 were then determined by western blotting. The results, in Fig. 4, show a number of interesting points. Firstly, HPV-16 E6 appears to degrade E6AP more strongly than HPV-18 E6, and this is in part dependent upon E6AP catalytic activity. These results are in agreement with previous studies (Kao et al., 2000). Most importantly however, both the wild type and mutant E6AP significantly increase the levels of both HPV-16 and HPV-18 E6 expression. These results demonstrate that E6AP contributes directly to the increased stability of HPV-16 and HPV-18 E6, and further, that this is independent of E6AP's catalytic activity.

Discussion

Previous studies have shown that E6AP is critical for the ability of E6 to target p53 for proteasome mediated degradation, which was largely believed to be a consequence of E6AP's ubiquitin ligase activity. However, there have been several reports that E6 can degrade proteins, albeit weakly, in the absence of functional E6AP, suggesting the existence of other routes by which E6 can degrade its substrate proteins (Camus et al. 2007; Massimi et al., 2008). Most importantly, it was also found (Kelley et al., 2005) that all the effects of E6 on cellular gene expression were mediated by E6AP. At first glance, these different studies appear irreconcilable. However, the results of the analysis presented here are perfectly consistent with all of the above reports. Thus, in the absence of E6AP, E6 levels are very low and the protein is rapidly degraded at the proteasome. Obviously, a by-product of this would be an apparent central requirement for E6AP in all of E6's biochemical activities, with loss of E6AP mimicking E6 ablation. However, in the light of the studies presented here, we can now conclude that the actual biochemical functions of E6, whilst needing E6AP for stability, are not necessarily E6AP-dependent with respect to substrate targeting and degradation.

These results raise a number of other interesting points. Firstly, it demonstrates that HPV-16 and HPV-18 E6 are regulated by the proteasome independently of E6AP, suggesting that other ubiquitin ligases may be involved in regulating E6 turnover. Whether these are involved in E6-induced degradation of some of its substrates is also an important question. These results also suggest that E6AP somehow blocks E6 degradation; this might be through masking E6 sites of ubiquitination or by altering E6 structure such that E6AP acts as a molecular chaperone. However, degradation of any putative E6 ubiquitin ligase by E6AP seems unlikely, since catalytically inactive E6AP is also capable of stabilising E6. This also suggests that this

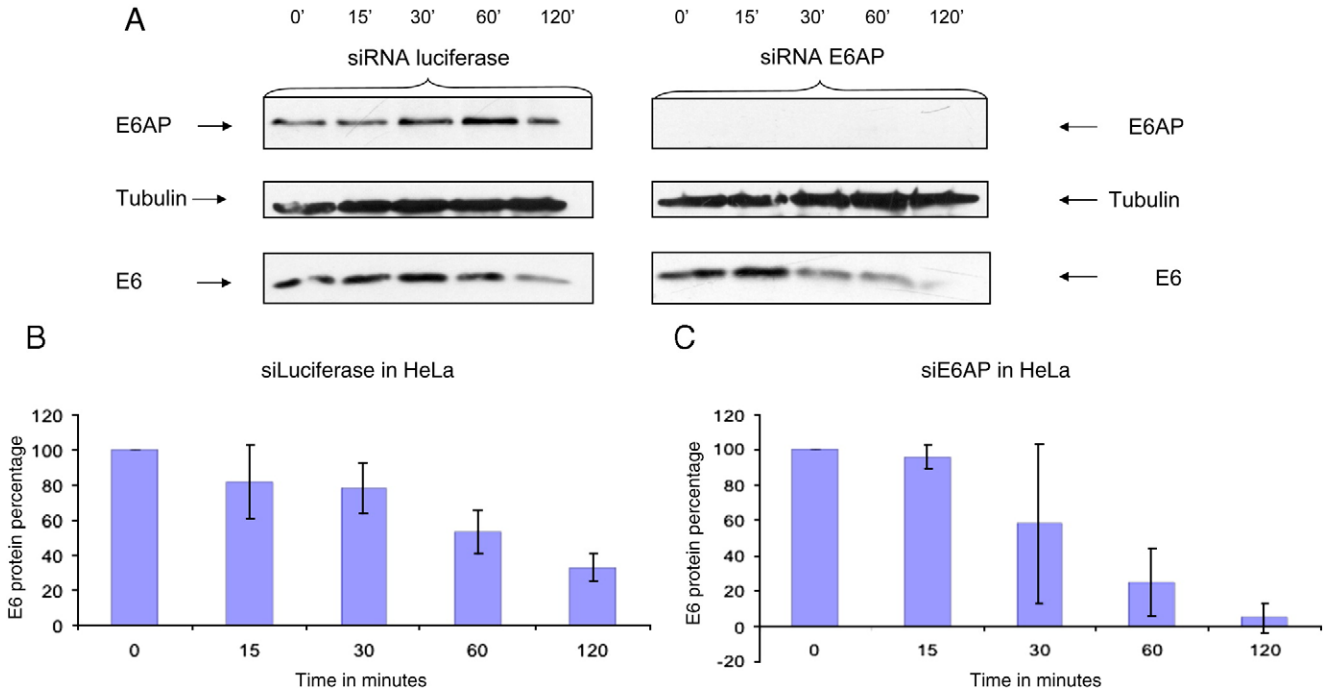

Fig. 3. E6 protein turnover is regulated by E6AP. A. HeLa cells were transfected with siRNA Luciferase or siRNA E6AP. 48 h post-transfection and prior to harvesting cells were treated with Cycloheximide for 5 different time points 0', 15', 30', 60' and 120'. Protein levels were detected using western blotting with anti-E6AP antibody, anti-tubulin antibody to monitor protein loading, and anti-18E6 antibody, followed by HRP-coupled anti-mouse antibody and ECL detection. Arrows indicate the positions of the E6AP, tubulin, and E6 proteins. The collated results from 3 independent experiments to measure E6 protein turnover in cells treated with siRNA Luciferase and siRNA E6AP are shown in panels B and C respectively. Band intensities were determined using the OptiQuant quantification program. E6 levels normalized to 100% at time 0. Standard deviations are also shown.

mutant, which can act in a dominant negative fashion with respect to E6 degradation of p53 (Beer-Romero et al., 1997; Talis et al., 1998), may alter E6 structure or inhibit ligase recruitment, rather than just acting to destabilise E6. These studies also suggest that variations in the levels of certain E6 substrate proteins, frequently observed in cervical tumour tissues (Cavatorta et al., 2004; Cooper et al., 1993), might actually be a reflection of alterations in E6AP levels or of the ability of E6AP to bind E6. Future studies will now aim to elucidate these aspects further and clarify how E6 mediates its degradation functions in the presence and absence of E6AP.

Materials and methods

Cell culture

HeLa and HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin–streptomycin (100 U/ml) and glutamine (300 μg/ml).

Plasmids

Untagged HPV-18 E6 and HPV-16 E6 pCDNA-3 expression plasmids have been described previously (Gardiol et al., 1999; Pim et al., 1994), as have the wild type and mutant E6AP (C→A) expression plasmids (Brimer et al., 2007).

Antibodies

The following antibodies were used: mouse monoclonal antibody against E6AP (1:500/BD Transduction Labs), anti-tubulin antibody (1:1000/Sigma Aldrich), mouse monoclonal anti-18E6 antibody (1:1000/Arbor Vita Corporation [N-terminus #399]), mouse monoclonal anti-16E6 antibody (1:1000/Arbor Vita Corporation [N-terminus #74 and C-terminus #813]), anti-FLAG monoclonal antibody (1/1000/Sigma) and anti-LacZ antibody (1/5000/Promega). Appropriate secondary antibodies conjugated to HRP were obtained from DAKO (1:1000).

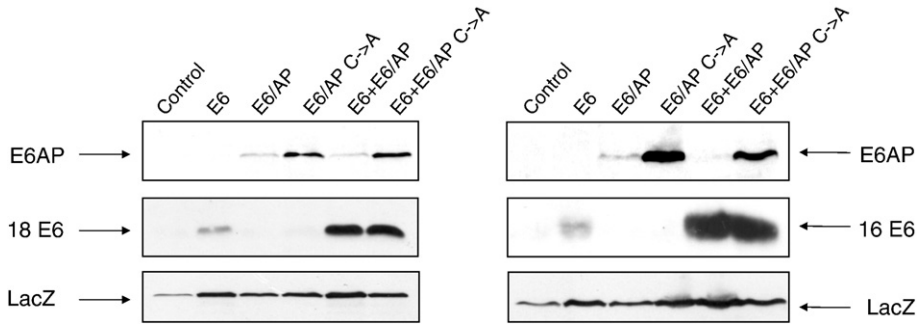

Fig. 4. Stabilisation of E6 by E6AP. HEK 293 cells were transfected with plasmids expressing HPV-16 and HPV-18 E6, together, as indicated, with FLAG-tagged wild type and a catalytically inactive mutant of E6AP (C→A) plus a LacZ expression plasmid. After 24 h the cells were harvested and the levels of E6AP were monitored using anti-FLAG antibody, and HPV-16 and HPV-18 E6 were monitored using the respective anti-E6 monoclonal antibody. Blots were stripped and re-probed for LacZ to control for transfection efficiency. Western blots were developed using appropriate HRP conjugated secondary antibody and ECL detection.

siRNA experiments

HeLa cells were seeded on 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with control siRNA against Luciferase (siLuc), or siRNA against HPV-18 E6/E7 sequences Dharmancon, or siRNA against E6AP sequences (Dharmacon and Santa Cruz). 72 h post-transfection cells were harvested and total cells extracts analysed by western blotting.

Western blotting

Cells were lysed in SDS lysis buffer. The whole cells extracts were analysed by SDS-PAGE and western blotting. For western blotting, 0.22 μm nitrocellulose membrane (Schleicher and Schuell) was used and membranes were blocked for 1 h at 37 °C in 10% milk/PBS followed by the incubation with the appropriate primary antibody diluted in 10% milk/0.5% Tween 20 for 2 h. After several washings with PBS 0.5% Tween 20, secondary antibodies conjugated with HRP (DAKO) in 10% milk/0.5% Tween 20 were incubated for 1 h. Blots were developed using Amersham ECL reagents according to the manufacturer's instructions.

Reverse Transcriptase (RT) RNA processing

Total RNA was extracted using the Tri reagent system (Sigma Aldrich) according to the manufacturer's protocol. The purified RNA was treated with RQ1 RNAse-free DNAse (Promega) according to the manufacturer's protocol to remove any plasmid DNA carried over during the RNA extraction. 2 μg of purified RNA was then annealed with random decamers and cDNAs generated with Reverse Transcriptase using a RETROScript Kit (Ambion) according to the manufacturer's instructions. The cDNAs generated from the RT step, and their control samples were amplified using HPV-18 E6 flanking oligos and Go-Taq Polymerase (Promega).

Half-life experiments

Prior to harvesting, cells were treated with Cycloheximide (1:2000/Sigma Aldrich) for different periods of time, after which the cells were harvested and subjected to western blot analysis.

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